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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

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INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/20641	27 July 2000	28 July 1999;28 January 2000; 28 April 2000

TITLE OF INVENTION
IMMUNOTHERAPY IN HIV INFECTED PERSONS USING VACCINES AFTER MULTI-DRUG TREATMENT

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST submission of items concerning a filing under 35 U.S.C. 371.**
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 36 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 37(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.2 and 35 U.S.C. 1.821 – 1.825.
18. ☐ A second copy of the published international application under 36 U.S.C.
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

EXPRESS MAIL NO. 827032437US; Declarations and Powers of Attorney of Genoveffa Franchini, Zdenek Hel, Gene Shearer, James Tartaglia, Janos Nacsa; Appointment of Associate Attorneys and Agents; Statement under 37 C.F.R. §3.73(b); Change of Correspondence Address; and Return Receipt Postcard.

I/S/ Application no (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Unknown 10/048072	PCT/US00/20641	15280-4003US

21. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(A) (1) – (5)):		
Neither international preliminary examination fee (37 CFR 1.492) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but International Search report prepared by the EPO of JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 – 20 =	0	x **$18.00**	$	
Independent claims	1 – 3 =	0	x **$84.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **280.00**	$	

TOTAL OF ABOVE CALCULATIONS =	$890.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½. +	$	
SUBTOTAL =	$890.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFT 1.492(f).	$	
TOTAL NATIONAL FEE =	$890.00	
Fee for recording the enclosed assignment (37 CFR 1.2(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$40.00	
TOTAL FEES ENCLOSED =	$930.00	
Amount to be refunded:	$	
charged:	$	

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 20-1430 in the amount of $ 930.00 to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 20-1430. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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NAME

44,879
REGISTRATION NUMBER

IMMUNOTHERAPY IN HIV INFECTED PERSONS USING VACCINES AFTER MULTI-DRUG TREATMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial Nos. 60/146,240, filed July 28, 1999; 60/178,989, filed January 28, 2000; and 60/200,445, filed April 28, 2000, each of which is incorporated by reference herein.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[Not Applicable]

FIELD OF THE INVENTION

This invention relates to an improved method of maintaining an immuno-protective response in persons infected with a retrovirus after highly active anti-retroviral therapy (HAART). Surpisingly, these patients demonstrate a $CD8^+$ response following HAART treatment.

SUMMARY OF THE INVENTION

The present invention is directed to a method of stimulating an efficient $CD8^+$ response in a human infected with an HIV or HTLV-1 retrovirus, who has a viral load of less than 10,000 viral copies, often 5,000 or 2,000 or less, per ml of plasma and a $CD4^+$ cell count that is often above 500 cells/ml, but can be above 400 cells/ml or 300 cells/ml; and who has been treated with one or more anti-viral agents, which contributed to a lower viral copy and higher $CD4^+$ cell count than before treatment. The method comprises administering a nucleic acid-based vaccine, which enters the cells and intracellularly produces HIV- or HTLV-1-specific peptides for presentation on the cell's MHC class I molecules in an amount sufficient to stimulate a protective $CD8^+$ response.

In a preferred embodiment the human has been treated with anti-viral agents, which resulted in the human having a viral load of less than 1,000 viral copies per ml of blood serum and a $CD4^+$ cell count that is above 500 cells/ml. The anti-viral agents can preferably comprise a combination of inhibitors of proteases and inhibitors of reverse transcriptase.

The method can use a vaccine that is a DNA based vaccine or that is an attenuated recombinant virus. A preferred virus is an attenuated pox virus, particularly NYVAC and ALVAC, attenuated vaccinia and canarypox viruses respectively. Other attenuated pox viruses such as MVA can also be used.

The vaccine can further comprise an adjuvant and may be adiminstered a second time. The vaccine can also comprise interleukin-2 (IL-2) and/or CD40 ligand in an amount that is sufficient to potentiate the $CD8^+$ response.

The method of the invention can be particularly useful for a person has who been infected with HIV and has demonstrated repeated and sustained proliferative T-cell responses to gp120 envelope protein or both gp 120 envelope and p24 gag antigen.

The person infected with HIV can be further tested by a skin test for a hypersensitive response to the p24 gag antigen or to the gp120 envelope antigen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Figure 1 shows the viral load and proliferative $CD4^+$ T-helper responses to p27 *gag* and gp120 in the infected, treated vaccinated macaques. The top panels display the kinetics of virus load in the plasma animals from groups A, B, and C. In group A two animals (641 and 642) did not respond to therapy and were not included in the analyses.

Figure 2: Figure 2 shows the $CD8^+$ response in the infected, treated, vaccinated animals.

Figure 3: Figure 3 shows p27-specific (Fig. 3a and 3c) and gp120-specific (Fig. 3b and 3d) T-cell proliferation in SIV-infected HAART-treated *Rhesus macaques* following administration of a single dose of ALVAC-SIV_{gpe}. (Fig. 3a and Fig. 3b) or

ALVAC (Fig. 3c and 3d). The arrows indicate time after SIV infection at which the animals were inoculated with vaccine.

Figure 4: Figure 4 shows the $CD3^+CD8^+$ T-cell responses detected in fresh peripheral blood mononuclear cells from SIV-infected, HAART-treated macaques inoculated with ALVAC-SIV_{gpe} (Fig. 4a) or ALVAC (Fig. 4b).

Figure 5: Figure 5 shows the induction of $CD8^+$ (Fig. 5a and 5b) and $CD4^+$ (Fig. 5c and 5d) T-cell responses following administration of a DNA vaccine to naïve macaques. DNA was administered at the times indicated by the arrow. The plasmids employed were pCMV-*gag* and pCMV-*env*, which are CMV expression plasmids expressing the gag and env genes, respectively.

Figure 6: Figure 6 shows the induction of $CD8^+$ (Fig. 6a and 6b) and $CD4^+$ (Fig. 6c and 6d) T-cell responses following administration of two inoculation of NYVAC-SIV-*gag-pol-env* to naïve macaques. The vaccine was at the times indicated by the arrows.

Figure 7: Figure 7 shows the administration schedule of NYVAC vaccine with and without interleukin-2 (IL-2) in HAART-treated macaques.

Figure 8: Figure 8 shows the amount of viral RNA (top panel) and the CD8+ and CD4+ proliferative responses, top and lower panel respectively, in a NYVAC-SIV-inoculated Rhesus macaque treated with IL-2. The horizontal bars indicate the length of treatment with anti-retroviral therapy (ART) and IL-2. Viral RNA levels in the plasmid (top panel) are indicated with open circles. The percent of $CD8^+CD3^+$ tetramer-binding cells is indicated in the top panel by the hatched vertical bars. ELISPOT assay results measuring the release of γ-interferon are shown by the black vertical bars. Proliferative responses to p27 gag and gp120 are indicated in the lower panel. IL-2 was administered daily by subcutaneous injection at a dose of 120,000 units.

Definitions

"Attenuated recombinant virus" refers to a virus that has been genetically altered by modern molecular biological methods, *e.g.* restriction endonuclease and ligase treatment, and rendered less virulent than wild type, typically by deletion of specific genes or by serial passage in a non-natural host cell line or at cold temperatures.

"Efficient $CD8^+$ response" is referred to as the ability of cytotoxic $CD8^+$ T-cells to recognize and kill cells expressing foreign peptides in the context of a major histocompatibility complex (MHC) class I molecule.

"Nonstructural viral proteins" are those proteins that are needed for viral production but are not necessarily found as components of the viral particle. They include DNA binding proteins and enzymes that are encoded by viral genes but which are not present in the virions. Proteins are meant to include both the intact proteins and fragments of the proteins or peptides which are recognized by the immune cell as epitopes of the native protein.

"Nucleic acid-based vaccines" include both naked DNA and vectored DNA (within a viral capsid) where the nucleic acid encodes B-cell and T-cell epitopes and provides an immunoprotective response in the person being vaccinated.

"Plasma" refers to the fraction of whole blood resulting from low speed centrifugation of EDTA- or heparin- treated blood.

"Pox viruses" are large, enveloped viruses with double-stranded DNA that is covalently closed at the ends. Pox viruses replicate entirely in the cytoplasm, establishing discrete centers of viral synthesis. Their use as vaccines has been known since the early 1980's (*see, e.g.* Panicali, D. *et al.* "Construction of live vaccines by using genetically engineered pox viruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin", *Proc. Natl. Acad. Sci. USA* 80:5364-5368, 1983).

A "retrovirus" is a virus containing an RNA genome and an enzyme, reverse transcriptase, which is an RNA-dependent DNA polymerase that uses an RNA molecule as a template for the synthesis of a complementary DNA strand. The DNA form of a retrovirus commonly integrates into the host-cell chromosomes and remains part of the host cell genome for the rest of the cell's life.

"Structural viral proteins" are those proteins that are physically present in the virus. They include the capsid proteins and enzymes that are loaded into the capsid

with the genetic material. Because these proteins are exposed to the immune system in high concentrations, they are considered to be the proteins most likely to provide an antigenic and immunogenic response. Proteins are meant to include both the intact proteins and fragments of the proteins or peptides which are recognized by the immune cell as epitopes of the native protein.

"Viral load" is the amount of virus present in the blood of a patient. Viral load is also referred to as viral titer or viremia. Viral load can be measured in variety of standard ways.

DETAILED DESCRIPTION

Introduction

This invention is a novel therapeutic modality for treating persons infected with a lymphotropic or immune destroying retrovirus. A physician presented with a patient whose immune system is compromised by retroviral infection can elect to treat that patient with a host of powerful antiviral agents including inhibitors of viral proteases and reverse transcriptase. This is known as highly active anti-retroviral therapy (HAART). The conventional HAART protocols are complex and difficult for patients to follow. The drugs also have a number of problematic side effects. In addition, these expensive and complicated treatments do not eliminate the virus, but merely hold the virus in check. If the patient is non-compliant, the viral counts rebound. Accordingly, for the vast majority of patients, a lifetime of drugs is advised.

This invention is the discovery that after HIV infection, HAART treatment can sufficiently restore a patient's immune system to effectively mount a $CD8^+$ response when a patient is provided with a $CD8^+$-inducing vaccine. This response can effectively maintain a low titer of virus and significantly reduce the patient dependency on HAART when the $CD8^+$-inducing vaccine is an HIV vaccine.. While some such vaccines have been suggested as useful for seropositive patients (U.S. Patent No. 5,863,542 column 18, lines 60-63), they are surprisingly effective for this subpopulation of seropositive patients and not for other seropositive patients.

Vaccines of use in this invention

Vaccines useful for the induction of $CD8^+$ T-cell responses comprise nucleic acid-based vaccines (delivered via a viral vector or directly as a DNA vaccine) that

provide for the intracellular production of viral-specific peptide epitopes that are presented on MHC Class I molecules and subsequently induce an immunoprotective cytotoxic T lymphocyte (CTL) response.

The invention contemplates single or multiple administrations of a nucleic acid-based vaccine as a direct DNA vaccine or as a recombinant virus vaccine, or both. This vaccination regimen may be complemented with administration of recombinant protein vaccines (*infra*), or may be used with additional vaccine vehicles.

Attenuated recombinant viral vaccines

Attenuated recombinant viruses that express retrovirus specific epitopes are of use in this invention. Attenuated viruses are modified from their wildtype virulent form to be either symptomless or weakened when infecting humans. Among the recombinant viruses of use are adenoviruses, adeno-associated viruses, retroviruses and poxviruses.

A recombinant, attenuated virus for use in this invention as a vaccine is a virus wherein the genome of the virus is defective with respect to a gene essential for the efficient production of, or essential for the production of, infectious virus. The mutant virus acts as a vector for an immunogenic retroviral protein by virtue of the virus encoding foreign DNA. This provokes or stimulates a cell-mediated $CD8^+$ response.

The virus is then introduced into a human vaccinee by standard methods for vaccination of live vaccines. A live vaccine of the invention can be administered at, for example, about 10^4 -10^8 organisms/dose, or 10^6 to 10^9 pfu per dose. Actual dosages of such a vaccine can be readily determined by one of ordinary skill in the field of vaccine technology.

The selection of the virus is not critical. Examples of viral expression vectors include adenoviruses as described in M. Eloit *et al*, "Construction of a Defective Adenovirus Vector Expressing the Pseudorabies Virus Glycoprotein gp50 and its Use as a Live Vaccine", *J. Gen. Virol.*, 71(10):2425-2431 (Oct., 1990).), adeno-associated viruses (*see, e.g.*, Samulski *et al.*, *J. Virol.* 61:3096-3101 (1987); Samulski *et al.*, *J. Virol.* 63:3822-3828 (1989)), papillomavirus, Epstein Barr virus (EBV) and Rhinoviruses (*see, e.g.*, U.S. Patent No. 5,714,374). Human parainfluenza viruses are also reported to be useful, especially JS CP45 HPIV-3 strain. The viral vector may be derived from herpes simplex virus (HSV) in which, for example, the gene encoding glycoprotein H (gH) has been inactivated or deleted. Other suitable viral vectors include retroviruses (*see, e.g.*, Miller, *Human Gene Ther.* 1:5-14 (1990); Ausubel *et al.*, *Current Protocols in Molecular Biology*).

The poxviruses are of preferred use in this invention. There are a variety of attenuated poxviruses that are available for use as a vaccine against HIV. These include attenuated vaccinia virus, cowpox virus and canarypox virus. In brief, the basic technique of inserting foreign genes into live infectious poxvirus involves a recombination between pox DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus as described in Piccini *et al.*, *Methods in Enzymology 153*, 545-563 (1987). More specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus described in U.S. Pat. Nos. 4,769,330, 4,722,848, 4,603,112, 5,110,587, and 5,174,993, the disclosures of which are incorporated herein by reference.

First, the DNA gene sequence encoding an antigenic sequence, such as a known T-cell epitope, is selected to be inserted into the virus. The sequence is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria.

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, *e.g.* chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively, gives a poxvirus modified by the presence, in a nonessential region of its genome, of foreign DNA sequences.

Attenuated recombinant pox viruses are a preferred vaccine. A detailed review of this technology is found in US Patent No. 5,863,542 which is incorporated by reference herein. Representative examples of recombinant pox viruses include ALVAC, TROVAC, NYVAC, and vCP205 (ALVAC-MN120TMG). These viruses were deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., 20852, USA: NYVAC under ATCC accession number VR-2559 on Mar. 6, 1997; vCP205 (ALVAC-MN120TMG) under ATCC accession number VR-2557 on Mar. 6, 1997;

TROVAC under ATCC accession number VR-2553 on Feb. 6, 1997 and, ALVAC under ATCC accession number VR-2547 on Nov. 14, 1996.

NYVAC is a genetically engineered vaccinia virus strain generated by the specific deletion of eighteen open reading frames encoding gene products associated with virulence and host range. NYVAC is highly attenuated by a number of criteria including: i) decreased virulence after intracerebral inoculation in newborn mice, ii) inocuity in genetically (nu^+/nu^+) or chemically (cyclophosphamide) immunocompromised mice, iii) failure to cause disseminated infection in immunocompromised mice, iv) lack of significant induration and ulceration on rabbit skin, v) rapid clearance from the site of inoculation, and vi) greatly reduced replication competency on a number of tissue culture cell lines including those of human origin.

TROVAC refers to an attenuated fowlpox that was a plaque-cloned isolate derived from the FP-1 vaccine strain of fowlpoxvirus which is licensed for vaccination of 1 day old chicks.

ALVAC is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia *et al.*, *AIDS Res Hum Retroviruses* 8:1445-7 (1992)). ALVAC has some general properties which are the same as some general properties of Kanapox. ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors. This avipox vector is restricted to avian species for productive replication. In human cell cultures, canarypox virus replication is aborted early in the viral replication cycle prior to viral DNA synthesis. Nevertheless, when engineered to express extrinsic immunogens, authentic expression and processing is observed *in vitro* in mammalian cells and inoculation into numerous mammalian species induces antibody and cellular immune responses to the extrinsic immunogen and provides protection against challenge with the cognate pathogen.

NYVAC, ALVAC and TROVAC have also been recognized as unique among all poxviruses in that the National Institutes of Health ("NIH")(U.S. Public Health Service), Recombinant DNA Advisory Committee, which issues guidelines for the physical containment of genetic material such as viruses and vectors, *i.e.*, guidelines for safety procedures for the use of such viruses and vectors which are based upon the pathogenicity of the particular virus or vector, granted a reduction in physical containment level: from BSL2 to BSL1. No other poxvirus has a BSL1 physical

containment level. Even the Copenhagen strain of vaccinia virus-the common smallpox vaccine-has a higher physical containment level; namely, BSL2. Accordingly, the art has recognized that NYVAC, ALVAC and TROVAC have a lower pathogenicity than any other poxvirus.

Another attenuated poxvirus of preferred use for this invention is Modified Vaccinia virus Ankara (MVA), which acquired defects in its replication ability in humans, as well as most mammalian cells, following over 500 serial passages in chicken fibroblasts (*see, e.g.*, Mayr *et al.*, *Infection* 3:6-14 (1975); Carrol, M. and Moss, B. *Virology* 238:198-211 (1997)). MVA retains its original immunogenicity and its variola-protective effect and no longer has any virulence and contagiousness for animals and humans. As in the case of NYVAC or ALVAC, expression of recombinant protein occurs during an abortive infection of human cells, thus providing a safe, yet effective, delivery system for foreign antigens.

The HIV antigen encoding DNA for insertion into these vectors is any that is known to be an effective antigen for protection against a retrovirus. For HIV these would include nucleic acid that can encode at least one of: HIV1gag(+ pro)(IIIB), gp120(MN)(+ transmembrane), nef(BRU)CTL, pol(IIIB)CTL, ELDKWA or LDKW epitopes, preferably HIV1gag(+ pro)(IIIB), gp120(MN) (+ transmembrane), two (2) nef(BRU)CTL and three (3) pol(IIIB)CTL epitopes; or two ELDKWA in gp120 V3 or another region or in gp160. The two (2) nef(BRU)CTL and three (3) pol(IIIB)CTL epitopes are preferably CTL1, CTL2, pol1, pol2 and pol3. In the above listing, the viral strains from which the antigens are derived are noted parenthetically.

Direct DNA delivery vaccines

As an alternative to a viral vaccine, the nucleic acid can also be directly introduced into the cells of a patient. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; and WO 98/04720. Examples of DNA-based delivery technologies include, "naked DNA", facilitated (bupivicaine, polymers, peptide-mediated) delivery, and cationic lipid complexes or liposomes. The nucleic acids can be administered using ballistic delivery as described, for instance, in Fynan *et al.*, *Proc Natl Acad Sci U S A.* 90:11478-82 (1993) and U.S. Patent No. 5,204,253 or pressure (*see, e.g.*, U.S. Patent No. 5,922,687). Using this technique, particles comprised solely of DNA are administered, or in an

alternative embodiment, the DNA can be adhered to particles, such as gold particles, for administration.

As is well known in the art, a large number of factors can influence the efficiency of expression of antigen genes and/or the immunogenicity of DNA vaccines. Examples of such factors include the reproducibility of inoculation, construction of the plasmid vector, choice of the promoter used to drive antigen gene expression and stability of the inserted gene in the plasmid.

Any of the conventional vectors used for expression in eukaryotic cells may be used for directly introducing DNA into tissue. Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of such promoters as the SV40 early promoter, SV40 later promoter, metallothionein promoter, human cytomegalovirus promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Therapeutic quantities of plasmid DNA can be produced for example, by fermentation in *E. coli*, followed by purification. Aliquots from the working cell bank are used to inoculate growth medium, and grown to saturation in shaker flasks or a bioreactor according to well known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins. If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods.

Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffer saline (PBS). This formulation, known as "naked DNA," is particularly suitable for intramuscular (IM) or intradermal (ID) administration.

To maximize the immunotherapeutic effects of plasmid DNA vaccines, alternative methods for formulating purified plasmid DNA may be desirable. A variety of methods have been described, and new techniques may become available. Cationic lipids can also be used in the formulation (*see, e.g.*, as described by WO 93/24640; Mannino & Gould-Fogerite, *BioTechniques* 6(7): 682 (1988); U.S. Pat No. 5,279,833; WO 91/06309; and Felgner, *et al., Proc. Nat'l Acad. Sci. USA* 84:7413

(1987). In addition, glycolipids, fusogenic liposomes, peptides and compounds referred to collectively as protective, interactive, non-condensing compounds (PINC) could also be complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types.

Selection of an HIV specific epitope.

Highly antigenic epitopes for provoking an immune response selective for a specific retroviral pathogen are known. The retrovirus, HIV is a major problem in the United States and in the world. With minor exceptions, the following discussion of HIV epitopes is applicable to other retroviruses except for the differences in sizes of the respective viral proteins. HIV-specific epitopes fall into two major categories, structural and non-structural proteins. Epitopes can be selected from either or both groups of proteins. Structural proteins are a physical part of the virion. Non-structural proteins are regulatory proteins. The envelope is a preferred source of epitopes and gp160, 120 and 41 are sources of immunoprotective proteins. Both B and T cell epitopes have been described in the literature and can be used. Peptides selected from the V3 loop of the HIV envelope proteins are of preferred use. In addition other structural proteins have been reported to be immunoprotective including p41, p17 and the gag protein. Non-structural genes include the *rev, tat, nef, vif*, and *vpr* genes.

Patients

A preferred patient population of retrovirally infected persons are those that exhibit repeated and sustained proliferative T-cell responses to envelope epitopes, *e.g.*, HIV gp120. More preferred are those patients that also respond to the *gag* epitopes, *e.g.* HIV p24. Typically these patients are identified by measuring the ability of their lymphocytes to proliferate in responses to highly purified antigen. In brief, peripheral blood monocytes (PBMC) are collected and cultured in the absence of IL-2 and in the presence of 10 μg of highly purified antigen. After four days the cultures are harvested and proliferation is measured by uptake of radioactive thymidine.

An alternative means of identifying these patients is to use a skin test. Skin tests involve the detection of a delayed type hypersenstive response (DTH) by means of injecting or scratching antigen beneath the surface of the skin. The reaction is measured by the ability or inability of a patient to exhibit hypersensitive response to an aqueous solution of a gp120

or p24 antigen. Approximately, 1-20 μg is applied. The reaction is determined by measuring wheal sizes from about 24 to about 72 hours after administration of a sample, and more preferably from about 48 hours to about 72 hours after administration of a sample. Preferred wheal sizes for evaluation of the hypersensitivity of an animal range from about 16 mm to about 8 mm, more preferably from about 15 mm to about 9 mm, and even more preferably from about 14 mm to about 10 mm in diameter.

Highly Active Anti-Retroviral Therapy (HAART)

Antiviral retroviral treatment involves the use of two broad categories of therapeutics. They are reverse transcriptase inhibitors and protease inhibitors. There are two type of reverse transcriptase inhibitors: nucleoside analog reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. Both types of inhibitors block infection by blocking the activity of the HIV reverse transcriptase, the viral enzyme that translates HIV RNA into DNA which can later be incorporated into the host cell chromosomes.

Nucleoside and nucleotide analogs mimic natural nucleotides, molecules that act as the building blocks of DNA and RNA. Both nucleoside and nucleotide analogs must undergo phosphorylation by cellular enzymes to become active; however, a nucleotide analog is already partially phosphorylated and is one step closer to activation when it enters a cell. Following phosphorylation, the compounds compete with the natural nucleotides for incorporation by HIV's reverse transcriptase enzyme into newly synthesized viral DNA chains, resulting in chain termination.

Examples of anti-retroviral nucleoside analogs are: AZT, ddI, ddC, d4T , and 3TC in combination with AZT and Combivir.

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are a structurally and chemically dissimilar group of antiretroviral compounds. They are highly selective inhibitors of HIV-1 reverse transcriptase. At present these compounds do not affect other retroviral reverse transcriptase enzymes such as hepatitis viruses, herpes viruses, HIV-2, and mammalian enzyme systems. They are used effectively in triple-therapy regimes. Examples of NNRTIs are Delavirdine and Nevirapine which have been approved for clinical use in combination with nucleoside analogs for treatment of HIV-infected adults who experience clinical or immunologic deterioration. A detailed review can be found in "Nonnucleoside Reverse Transcriptase Inhibitors" *AIDS Clinical Care* (10/97) Vol. 9, No. 10, p. 75.

Proteases inhibitors are compositions that inhibit HIV protease, which is virally encoded and necessary for the infection process to proceed. Clinicians in the United States have a number of clinically effective proteases to use for treating HIV-infected persons. These include: SAQUINAVIR (Invirase); INDINAVIR (Crixivan); and RITONAVIR (Norvir).

$CD4^+$ T cell counts

To assess a patient's immune system before antiviral treatment and after treatment as well as to determine if the claimed vaccine regimen is working it is important to measure $CD4^+$ T cell counts. A detailed description of this procedure was published by Janet K.A. Nicholson, Ph.D *et al.* 1997 *Revised Guidelines for Performing $CD4^+$ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV)* in *The Morbidity and Mortality Weekly Report*, 46(RR-2):[inclusive page numbers], Feb 14, 1997, Centers for Disease Control.

In brief, most laboratories measure absolute $CD4^+$ T-cell levels in whole blood by a multi-platform, three-stage process. The $CD4^+$ T-cell number is the product of three laboratory techniques: the white blood cell (WBC) count; the percentage of WBCs that are lymphocytes (differential); and the percentage of lymphocytes that are $CD4^+$ T-cells. The last stage in the process of measuring the percentage of $CD4^+$ T-lymphocytes in the whole-blood sample is referred to as "immunophenotyping by flow cytometry.

Immunophenotyping refers to the detection of antigenic determinants (which are unique to particular cell types) on the surface of WBCs using antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome (e.g., phycoerythrin [PE] or fluorescein isothiocyanate [FITC]). The fluorochrome-labeled cells are analyzed by using a flow cytometer, which categorizes individual cells according to size, granularity, fluorochrome, and intensity of fluorescence. Size and granularity, detected by light scattering, characterize the types of WBCs (*i.e.*, granulocytes, monocytes, and lymphocytes). Fluorochrome-labeled antibodies distinguish populations and subpopulations of WBCs.

Systems for measuring $CD4^+$ cells are commerically available. For example Becton Dickenson's FACSCount System automatically measure absolutes $CD4^+$, $CD8^+$, and $CD3^+$ T lymphocytes. It is a self-contained system, incorporating instrument, reagents, and controls.

Viral titer

There are a variety of ways to measure viral titer in a patient. A review of the state of this art can be found in the *Report of the NIH To Define Principles of Therapy of HIV Infection* as published in the ;*Morbidity and Mortality Weekly Reports*, April 24, 1998, Vol 47, No. RR-5, Revised 6/17/98. It is known that HIV replication rates in infected persons can be accurately gauged by measurement of plasma HIV concentrations.

HIV RNA in plasma is contained within circulating virus particles or virions, with each virion containing two copies of HIV genomic RNA. Plasma HIV RNA concentrations can be quantified by either target amplification methods (*e.g.*, quantitative RT polymerase chain reaction [RT-PCR], Amplicor HIV Monitor assay, Roche Molecular Systems; or nucleic acid sequence-based amplification, [NASBA®], NucliSens™ HIV-1 QT assay, Organon Teknika) or signal amplification methods (*e.g.*, branched DNA [bDNA], Quantiplex™ HIV RNA bDNA assay, Chiron Diagnostics). The bDNA signal amplification method amplifies the signal obtained from a captured HIV RNA target by using sequential oligonucleotide hybridization steps, whereas the RT-PCR and NASBA® assays use enzymatic methods to amplify the target HIV RNA into measurable amounts of nucleic acid product. Target HIV RNA sequences are quantitated by comparison with internal or external reference standards, depending upon the assay used.

Measurements of $CD8^+$ Responses

$CD8^+$ T-cell responses can be measured, for example, by using tetramer staining of fresh or cultured PBMC (*see, e.g.*, Altman, J. D. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10330, 1993; Altman, J. D. *et al.*, *Science* 274:94, 1996), or γ-interferon release assays such as ELISPOT assays (*see, e.g.*, Lalvani, A. *et al.*, *J. Exp. Med.* 186:859, 1997; Dunbar, P. R. *et al.*, *Curr. Biol.* 8:413, 1998; Murali-Krishna, K. *et al.*, *Immunity* 8:177, 1998), or by using functional cytotoxicity assays. Each of these assays are well-known to those of skill in the art. For example, a cytotoxicity assay can be performed as follows.

Briefly, peripheral blood lymphocytes from patients are cultured with HIV peptide epitope at a density of about five million cells/ml. Following three days of culture, the medium is supplemented with human IL-2 at 20 units/ml and the cultures

are maintained for four additional days. PBLs are centrifuged over Ficoll-Hypaque and assessed as effector cells in a standard ^{51}Cr-release assay using U-bottomed microtiter plates containing about 10^4 target cells with varying effector cell concentrations. All cells are assayed twice. Autologous B lymphoblastoid cell lines are used as target cells and are loaded with peptide by incubation overnight during ^{51}Cr labeling. Specific release is calculated in the following manner: (experimental release-spontaneous release)/(maximum release-spontaneous release) x 100. Spontaneous release is generally less than 20% of maximual release with detergent (2% Triton X-100) in all assays.

Formulation of Vaccines and Administration

The administration procedure for recombinant virus or DNA is not critical. Vaccine compositions (*e.g.*, compositions containing the poxvirus recombinants or DNA) can be formulated in accordance with standard techniques well known to those skilled in the pharmaceutical art. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration.

For example, NYVAC-HIV or other attenuated pox virus vaccines such as ALVAC-HIV or MVA-HIV, is inoculated, more than once, by the intramuscular route at a dose of about 10^8 pfu per inoculation, for a patient of 170 pounds. The vaccine can be delivered in a physiologically compatible solution such as sterile PBS in a volume of, *e.g.*, one ml. The dose can be proportional to weight.

The compositions can be administered alone, or can be co-administered or sequentially administered with other immunological, antigenic, vaccine, or therapeutic compositions. Such compositions can include other agents to potentiate or broaden the immune response, *e.g.*, IL-2 or CD40 ligand, which can be administered at specified intervals of time, or continuously administered (*see, e.g.*, Smith *et al., N Engl J Med* 1997 Apr 24;336(17):1260-1; and Smith, *Cancer J Sci Am.* 1997 Dec;3 Suppl 1:S137-40). For example, IL-2 can be administered in a broad range, *e.g.*, from 10,000 to 1,000,000 or more units. Administration can occur continuously following vaccination. Often, low doses, *e.g.* 100,000 to 200,000, often 120,000, 150,000 or 170,000, units of IL-2 can be particularly useful.

Other compositions that can be co-administered can include purified antigens from immunodeficiency virus or antigens that are expressed by a second recombinant vector system which is able to produce other therapeutic compositions. Such compositions can include a recombinant poxvirus which expresses other immunodeficiency antigens or biological response modifiers (*e.g.* cytokines; co-stimulating molecules). Again, co-administration is performed by taking into consideration such known factors as the age, sex, weight, and condition of the particular patient, and, the route of administration.

DNA expression vectors for direct introduction of DNA into the patient tissue can additionally be complexed with other components such as peptides, polypeptides and carbohydrates. Expression vectors can also be complexed to particles or beads that can be administered to an individual, for example, using a vaccine gun.

The expression vectors are administered by methods well known in the art as described in Donnelly *et al.* (*Ann. Rev. Immunol.* 15:617-648 (1997)); Felgner *et al.* (U.S. Patent No. 5,580,859, issued December 3, 1996); Felgner (U.S. Patent No. 5,703,055, issued December 30, 1997); and Carson *et al.* (U.S. Patent No. 5,679,647, issued October 21, 1997), each of which is incorporated herein by reference. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector.

For example, naked DNA or polynucleotide in an aqueous carrier can be injected into tissue, such as muscle, in amounts of from 10 μl per site to about 1 ml per site. The concentration of polynucleotide in the formulation is from about 0.1 μg/ml to about 20 mg/ml.

Vaccines can be delivered via a variety of routes. Typical delivery routes include parenteral administration, *e.g.*, intradermal, intramuscular or subcutaneous routes. Other routes include oral administration, intranasal, and intravaginal routes.

The expression vectors of use for the invention can be delivered to the interstitial spaces of tissues of a patient (*see, e.g.*, Felgner *et al.*, U.S. Patent Nos. 5,580,859, and 5,703,055). Administration of expression vectors of the invention to muscle is a particularly effective method of administration, including intradermal and subcutaneous injections and transdermal administration. Transdermal administration, such as by iontophoresis, is also an effective method to deliver expression vectors of the invention to muscle. Epidermal administration of expression vectors of the invention can also be employed. Epidermal

administration involves mechanically or chemically irritating the outermost layer of epidermis to stimulate an immune response to the irritant (Carson *et al.*, U.S. Patent No. 5,679,647).

The vaccines can also be formulated for administration via the nasal passages. Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 10 to about 500 microns which is administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the active ingredient. For further discussions of nasal administration of AIDS-related vaccines, references are made to the following patents, US 5,846,978, 5,663,169, 5,578,597, 5,502,060, 5,476,874, 5,413,999, 5,308,854, 5,192,668, and 5,187,074.

Examples of vaccine compositions of use for the invention include liquid preparations, for orifice, *e.g.*, oral, nasal, anal, vaginal, etc. administration, such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (*e.g.*, injectable administration) such as sterile suspensions or emulsions. In such compositions the recombinant poxvirus, expression product, immunogen, DNA, or modified gp120 or gp160 can be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like.

The vaccines can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (*see, e.g.*, Felgner *et al.*, U.S. Patent No. 5,703,055; Gregoriadis, *Liposome Technology*, Vols. I to III (2nd ed. 1993). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like.

Liposome carriers can serve to target a particular tissue or infected cells, as well as increase the half-life of the vaccine. In these preparations the vaccine to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, *e.g.*, a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes

either filled or decorated with a desired immunogen of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the immunogen(s).

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka, *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

Example 1. Administration of NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ to SIV-infected, HAART-treated *Rhesus macaques*

The effectiveness of a highly attenuated Poxvirus vector as a therapeutic vaccine to enhance host-immune responses was investigated in the SIV_{251} Rhesus macaque model, which models HIV-1 infection in humans. The vaccine used for this study was a highly attenuated NYVAC-SIV *gag-pol-env* recombinant vaccine that was demonstrated to have efficacy as a preventative vaccine in earlier studies (Benson *et al.*, *J. Virol.* 72:4170-4182 (1998).

The study design included 24 animals which were divided into three groups, A, B, and C. All the animals were infected intraveneously with ten infectious doses of highly pathogenic SIV_{251} (Pal). Following SIV_{251} exposure, all twenty four

animals became infected; the peak of plasma viremia occurred at approximately two weeks and ranged between 10^7 and 10^9 copies of viral RNA/ml of plasma in the twenty four animals.

Two and a half weeks after infection, sixteen animals, those in groups A and B, received a HAART regimen that in a pilot study had reduced viremia to undetectable levels in 80% of macaques chronically infected with SIV_{251}. The HAART regimen included oral administration of two doses of Stavudine (1.2 mg/day), intravenous inoculation of DDI (10 mg/kg/day) and subcutaneous inoculation of PMPA (20 mg/kg/day). Animals in group C were not treated with drugs. Animals in groups A and B, but not C, experienced a significant decrease in viremia (Figure 1, top panels).

In the sixteen animals of groups A and B, HAART treatment was continued daily for 6 months. At weeks 10, 19, and 23 post-infection, the animals in group A received a placebo vaccine (non-recombinant NYVAC vector) and the animals in groups B and C received 10^8 pfu of NYVAC-SIV *gag-pol-env* vaccine. All twenty-four animals were monitored weekly for viral RNA copies/ml of plasma and biweekly for lymphoproliferativ responses (LPR) to highly purified native p27 *gag* and gp120 *env* SIV proteins.

In order to follow the $CD8^+$ T-cell responses, three MAMU-A*01 (*Macaca mulat*a equivalent of HLA class I A*01 (Kuroda *et al., J. Exp. Med.* 187:1373-1381, 1998) were included in each group. MAMU-A*01 animals are generally able to recognizes the immunodominant peptide 11c-m within the gag antigen of SIV. A tetramer binding assay was therefore used to directly quantitate $CD8^+$ T-cell responses *in vivo*. The tetramer, formed by four identical MAMU-A*01 molecules conjugated to the peptide 11c-m, was linked to fluorescent-labeled streptavidin and used to stain the $CD8^+$ T-cells in the blood of macaques that expressed the appropriate T cell receptor complex on their surface. The percentage of total $CD8^+/CD3^+$ staining with the peptide MAMU-A*-1 tetramer was measured in several consecutive time intervals in each of the MAMU-A*01 animals.

Staining of fresh or cultured PBMC with the MAMU A*01/peptide 11c-m tetramer was performed in all the nine MAMU A*01 animals included in the study (three in each group) following SIV_{251} infection and after the second NYVAC-SIV vaccination. MAMU-A*01/peptide-11c-m-tetramer-staining $CD8^+$ T-cells (ranging from 0.8 to 4.6%) were induced by SIV infection in all nine animal within the first

month after viral exposure, and the $CD8^+$ T-cell population was expanded (up to values of approximately 70%) *in vitro* following specific peptide 11c-m stimulation. At two months after SIV_{251} exposure, the infected macaques seroconverted to SIV_{251} antigens, including the animals in groups A and B that were treated with HAART. Measurements of LPR to gp120 and p27 *gag* was consistently negative in all twenty four animals within the first four weeks following viral infection.

The frequency and extent of $CD4^+$ T-helper responses in HAART-treated animals is increased by NYVAC-SIV vaccination.

As stated above, acute infection by SIV_{251} was associated with the absence of a proliferative response to both p27 *gag* and gp120 *env*. However, responses to p27 *gag* appeared in HAART-treated animals at approximately 10 weeks after infection and, these responses were more frequent in animals of group A than in animals of group C. No difference in the LPR to gp120 was observed between these two groups (Figure 1, middle and lower panels).

This notion was further supported by the finding that two animals (647 and 655) in group B failed to respond to therapy, maintained high virus load, and did not develop CD4+ T-cell proliferative response following NYVAC-SIV vaccination.

Further corroborating this notion, two animals in group C that naturally controlled viremia developed LPR following NYVAC-SIV vaccination. Thus, it appears that $CD4^+$ T-helper memory responses are induced inefficiently by NYVAC-SIV animals with high viremia. Several factors may contribute to this finding: $CD4^+$ T-cells are already activated *in vivo* and do not further proliferate *in vitro* in LPR assays and/or the vaccine-induced memory cells become targets for SIV infection and die upon further antigen stimulation. These data provide the first evidence that a highly attenuated live recombinant poxvirus vector vaccine can induce and boost sustained $CD4^+$ helper immune response in the context of a pharmacologically controlled lentiviral infection.

*NYVAC-SIV vaccination increases $CD8^+/CD3^+$ MAMU-A*01-tetramer-positive cells only in HAART-treated animals.*

SIV infection induced a large number of $CD8^+$ T-cells that bound the MAMU-A*01 tetramer. This response by week four was reduced in most animals. Following the second and third NYVAC-SIV vaccinations, a high percentage of $CD8^+/CD3^+$ T-

cells bound tetramers in the fresh PBMC of all MAMU-A*01 animals in group B, but in none of the animals in groups C (Figure 2). The specificity of tetramer staining was shown in parallel experiments using PBMC from animals with a different haplotype as well as by the expansion of the cells from the nine MAMU-A*01 animals *in vitro* following peptide-11c-m-specific stimulation. Peptide 11c-m presented in the context of the MAMU-A*01 haplotype is recognized by $CD8^+$ T-cells with cytolytic activity. (In Figure 2, the top panel shows the results obtained in the three MAMU-A*01 animals from group A; middle panel, from group B; and bottom panel, from group C. The percentage of MAMU-A*01/peptide-11c-m-tetramer-staining cells within the first 4 weeks was evaluated using only α-$CD8^+$ antibodies as a T cell marker whereas the data presented from weeks 19 through 29 were obtained using simultaneously α-CD-3+ and α-$CD8^+$ antibodies in conjunction with the MAMU-A*01/peptide-11c-m tetramers. For some of the MAMU-A*01-positive animals, CTL activity obtained in cultured PBMC [weeks 19 and 20] or fresh PBMC [week 23] is also presented. The numbers in the abscissa represent the effector-target ratio of the CTL assay system.)

To assess whether the detection of MAMU-A*01/peptide-11c-m-tetramer-staining $CD8^+/CD3^+$ T-cells in the PBL of macaques was associated with CTL activity, cytotoxicity assays were performed using homologous B cells from each animal pulsed with peptide 11 c-m. It was shown that CTL activity was measured after *in vitro* stimulation of $CD8^+$ T-cells in all animals tested at weeks 19 and 20, although the extent of killing did not correlate with the percentage of tetramer-staining cells (Figure 2). Most notably, CTL assays performed on fresh $CD8^+$ T cells at day 23 demonstrated significant CTL activity in group B animals, confirming the CTL functional activity of the high number of circulating $CD8^+$ tetramer-staining cells in the blood of these animals (Figure 2). Thus, NYVAC-SIV vaccination induced high levels of $CD8^+$ responses only in animals in which viral replication was suppressed by therapy.

Delayed T-cell hypersensitivity (DTH) to viral p27 gag

Vaccines able to induce T-cell-mediated immunity are often able to induce DTH. To assess whether any of the animals vaccinated with NYVAC-SIV developed this response, either 1 or 10 ug of highly purified SIV p27 or HTLV-I p24, as controls, were inoculated

intradermally in animals in groups B and C. DTH reactivity was considered positive when a thickness of more than 10 mm manifested at 72 h postinoculation. Only three animals in group B and two animals in group C fulfilled the requirement for DTH positivity.

The data presented in this example demonstrated that inoculations of NYVAC-SIV following HAART greatly increased the frequency, extent, and duration of those responses in animals in which viremia was efficiently suppressed, indicating that the ability to detect vaccine-induced $CD4^+$ T-cell helper responses was strictly dependent on the level of viral replication in the host. Similarly, NYVAC-SIV vaccination induced significant expansion of the number of $CD8^+/CD3^+$ MAMU-A*01 cells specific for an immunodominant SIV *gag* peptide only in animals treated effectively with antiviral therapy. Following therapy suspension, NYVAC-SIV-vaccinated animals were able to control viremia better than animals treated with antiviral therapy alone. These data demonstrate that vaccination can further induce both $CD4^+$ and $CD8^+$ T-cell responses in SIV-infected macaques.

Example 2. Immunization of a person infected with HIV.

A 35 year old male patient is seropositive for HIV and has a viral count in his plasma of 15000 copies per milliliter and a $CD4^+$ count of 300. The patient is treated with a cocktail of antiviral agents which consist of: two nucleotide inhibitors and one protease inhibitor (Zaduvidine [three time a day], Lamivudine [two times a day], and Nesinavir [three times day] at preformulated doses. Typically, antiretroviral therapy is considered effective if a decrease of at least one log of the plasma viral RNA is observed. The plasma RNA load as well as the $CD4^+$ T-cell count in the blood will be measured monthly.

After six months the patient is re-evaluated and is determined to have a viral count in his plasma of 1500 copies per milliliter or less and a $CD4^+$ count of 500. He is then injected with an attenuated pox virus vector NYVAC carrying the following viral peptides: *gag*, *pro*, gp120-TM, *pol* and *nef* string of CTL epitopes. The injection comprises 10^8 pfu of the pox virus.

The patient's immune responses is evaluated (CD4+ proliferative response; cytotoxic CD8+ T-cell activity, etc.) and a decision is made as to whether and when to immunize again. Typically, a maximum of three to four immunizations with NYVAC-HIV-1 is considered. This regimen could be followed by three to four immunizations with ALVAC-HIV-1 (carrying a similar HIV-I genetic content) and, if

necessary, an additional regimen of DNA-only *i.e.,* DNA that is not in a viral vector, immunization could follow. NYVAC-HIV-1 followed by ALVAC-HIV-1 is effective in inducing immunoresponses in chimpanzees. Similarly, immunization regimens as follows have been shown to be effective: NYVAC followed by DNA and ALVAC followed by DNA. Thus, a vaccination regimen including all of the above vaccines may be used.

Often, the vaccine regimen is administered with IL-2, preferably at low doses such as 100,000 to 200,000 units of IL-2 administered daily. $CD40^+$ ligand can also be included in the treatment protocol, either by itself or administerd in conjunction with the IL-2 treatment.

Example 3. Administration of ALVAC-SIV_{gpe} to SIV-infected, HAART-treated *Rhesus macaques*

The effectiveness of a highly attenuated ALVAC vector as a therapeutic vaccine to enhance host-immune responses in HAART-treated animals was investigated in the SIV_{251} *Rhesus macaque* model, which models HIV-1 infection in humans. The study includes 16 macaques inoculated with the SIV_{251} virus and treated with the HAART regimen as described in Example 1 at day 15 and thereafter. Of those macaques, 8 are immunized with a total of 3 doses of 10^8 pfu of the mock-vaccine ALVAC vector (group D) and the remaining 8 (group E) with a recombinant ALVAC-SIV-*gag-pol-env* vector (ALVAC-SIV_{gpe}), which is analogous to the NYVAC vector of Example 1.

The data obtained after a single dose of the ALVAC-SIV_{gpe} vaccine indicated that the ALVAC-SIV_{gpe} vaccine is able to boost $CD4^+$ T-cell responses against the p27 Gag protein as well as to the gp120 Env forefront of the vaccine (see Figure 3). In addition, a specific $CD8^+$ T-cell response detected using the MAMU A*01/peptide 11c-m tetramer reagent (*see, e.g.*, Example 1) was also boosted by the ALVAC-SIV_{gpe} (Figure 4), and further, those $CD8^+$ T-cells could be expanded in culture. The mock-vaccinated animals did not experience an expansion of these immune responses.

Thus, ALVAC-SIV_{gpe} is immunogenic in animals undergoing HAART therapy. An ALVAC vaccine analogous to the NYVAC vaccine of Example 2 can similarly be employed to treat HIV-infected individuals as described in Example 2 who are undergoing HAART.

Example 4. MVA-SIV-*gag-pol-env* immunization of SIV-infected macaques.

The effectiveness of an MVA-SIV vaccine was evaluated using methodology analogous to that used to evaluate NYVAC and ALVAC SIV *gag-pol-env* vaccines. The CD8+ and $CD4^+$ responses were determined in SIV_{251}-infected macaques that were able to control viremia, *i.e.*, the CD4+ T-cell counts were above 500, following administrations of a single dose of 10^8 pfu of MVA-SIV-*gag-pol-env* recombinant vaccine. The results showed that both $CD4^+$ and $CD8^+$ responses could be expanded in infected animals.

Thus, MVA-SIV-*gag-pol-env* is also immunogenic in animals and can be used in patients undergoing HAART therapy.

Example 5. Comparison of the Immunogenicity of DNA and NYVAC vaccines.

A vaccine regimen comprising administration of DNA alone or DNA in combination with NYVAC-SIV or ALVAC-SIV, or administration of a combination of NYVAC-SIV and ALVAC-SIV is also effective in continuous boost of the immune response in SIV_{251}-infected animals.

A study conducted in parallel compared 2 inoculations of 10^8 pfu of NYVAC-SIV_{gpe} and 3 inoculations of DNA, which was administered using 4mg intramuscularly and 1 mg intradermally of each plasmid, in naïve animals. The DNA vaccine induced $CD4^+$ and $CD8^+$ T-cell responses that were equivalent to those induced by a NYVAC-SIV_{gpe} vaccine (Figures 5 and 6). ALVAC-SIV_{gpe} was at least as immunogenic as NYVAC-SIV_{gpe} (*see, e.g.*, Example 1) and NYVAC-SIV_{gpe} was as immunogenic as DNA. Thus, all three vaccines either alone or in various combinations can be used in HIV-I-infected individuals.

ALVAC-SIV_{gpe} was also able to induce both $CD4^+$ and $CD8^+$ T-cell response in chronically infected animals ($CD4^+$ T-cell range 50-900) and was able to suppress viremia in the absence of HAART. These animals, which had been previously vaccinated with a NYVAC-SIV_{gpe}-based vaccine, appear to model long-term progressor HIV-I-infected individuals. The vaccines of the invention can therefore be used alone or in various interchangeable combinations in early infection as well as late infection in individuals in which viremia is controlled pharmacologically or otherwise.

Example 6. Therapeutic Vaccination with NYVAC and IL-2.

This examples demonstrates the ability of immunomodulatory molecules, *e.g.* IL-2, to further expand both $CD4^+$ and $CD8^+$ T-cell responses induced

by the NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccine and increase the breadth of the host response to the virus.

SIV$_{251}$-infected HAART-treated Rhesus macaques (Figure 7) were vaccinated intramuscularly with NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ with or without simultaneous and continued daily treatment with IL-2 (120,000 IU) administered subcutaneously. A control group of animals was treated with IL-2 and mock-vaccinated (NYVAC nonrecombinant vector). All 15 macaques in the study responded to HAART (10 mg/kg/day DDI, intravenously; 2.4 mg/kg/day Stavudine, orally; 10 mg/kg/day PMPA, subcutaneously) and in 13 animals viremia was suppressed below 5 x 10^3 copies/ml within the first 4 weeks of treatment. Viremia in the remaining 2 animals became undetectable by weeks 6 and 8 of treatment. Proliferative responses to p27 Gag and gp120 were increased by NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccination up to three- and twelve-fold, respectively, regardless of IL-2 treatment, indicating that either IL-2 does not increase bulk proliferative response or that the assay was not sensitive enough to measure subtle variation in the antigen-specific CD4$^+$ T-helper response.

Approximately half of the animals in the study were genetically selected as carriers of the Mamu-A*01 molecules. CTL CD8$^+$ T-cell responses to SIV$_{251}$ were therefore measured by ELISPOT using several purified SIVmac$_{239}$ nonamer peptides and their corresponding Mamu-A*01 tetramers. The *ex vivo* PBMC of all SIV$_{251}$-infected Mamu-A*01 animals recognized the immunodominant p11C,C→M peptide and produced γ-interferon (γ-INF) following *in vitro* stimulation. This response was further expanded following immunization with NYVAC-SIV$_{gag\text{-}pol\text{-}env}$. While IL-2 did not expand the number of γ-INF-producing cells in response to the p11c, C→M peptide in mock-vaccinated animals, the expansion of this response was higher in the NYVAC-SIV$_{gag\text{-}pol\text{-}env}$–treated macaques that also received IL-2 than in those that received NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ alone. In contrast, IL-2 *per se* appeared to expand the immune response to two other immunodominant epitopes within the SIV tat and vif proteins in treated animals. (Vaccination with NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ did not further expand these responses as these antigens are not included in the vaccine.) Moreover, the CD8$^+$ T-cell responses to 2 subdominant epitopes within the Gag and Env proteins of SIV were clearly expanded following NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccination in macaques that received simultaneous and continuous IL-2 treatment. Thus, the administration of low-dose IL-2 in conjunction with vaccination with the

highly attenuated NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccine potentiated and broadened CD8$^+$ T-cell functional responses to SIV$_{251}$.

IL-2 can be used with the vaccine to control viremia after antiretroviral therapy interruption

A HAART-treated macaque was inoculated with NYVAC-SIV*gag-pol-env* at the intervals shown in Figure 8. The macaque also recived low dose IL-2, *i.e.*, 120,000 units daily administered subcutaneously. This animal exhibited expanded CD8$^+$ (top panel, Figure 8) and CD4+ proliferative (lower panel, Figure 8) responses. Furthermore, as shown in the top panel of Figure 8, transient viral rebound occurred following interruption of HAART treatment and after suspension of the IL2 treatment. Thus, administration of IL-2 in conjunction with vaccination can contribute to the control of viremia after interruption of antiretroviral therapy.

WHAT IS CLAIMED IS:

1. A method of stimulating an efficient $CD8^+$ response in a human infected with a an HIV or HTLV-1 retrovirus said method comprising:

administering to the human, a nucleic acid-based vaccine, which enters the cells of the human and intracellularly produces HIV- or HTLV-1-specific peptides for presentation on the cell's MHC class I molecules

where said peptides are presented in an amount sufficient to stimulate a protective $CD8^+$ response and

where said human

i. has a viral load of less than 10,000 viral copies per ml of plasma and a $CD4^+$ cell count of above 500 cells/ml, and

ii. has been treated with one or more anti-viral agents, which contributed to a lower viral copy and higher $CD4^+$ cell count than before treatment.

2. A method of claim 1 wherein the human has been treated with anti-viral agents, which resulted in the human having a viral load of less than 1,000 viral copies per ml of blood serum and a $CD4^+$ cell count of above 500 cells/ml.

3. A method of claim 2 wherein the anti-viral agents comprise a combination of protease inhibitors and inhibitors of reverse transcriptase.

4. A method of claim 1 wherein the vaccine is a DNA based vaccine.

5. A method of claim 1 wherein the vaccine is an attenuated recombinant virus.

6. A method of claim 5 wherein the vaccine is an attenuated pox virus.

7. A method of claim 6 wherein the attenuated pox virus is selected from the group consisting of NYVAC and ALVAC.

8. A method of claim 6 wherein the attenuated pox virus is MVA.

9. A method of claim 1 where the vaccine is administered a second time.

10. A method of claim 1 wherein the HIV- or HTLV-1-specific peptides are structural viral peptides

11. A method of claim 1 wherein the HIV- or HTLV-1-specific peptides are non-structural viral peptides.

12. A method of claim 1 wherein the vaccine further comprises an adjuvant.

13. A method of claim 1 further comprising administering interleukin 2 or CD40 ligand in an amount sufficient to potentiate the $CD8^+$ response.

14. A method of claim 1 where the person has been infected with HIV and has demonstrated repeated and sustained proliferative T-cell responses to gp120 envelope protein.

15. A method of claim 14 where the person has demonstrated repeated and sustained proliferative T-cell responses to p24 gag antigen.

16. A method of claim 1 where the person is infected with HIV and is further tested by a skin test for a hypersensitive response to p24 gag antigen.

17. A method of claim 1 where the person is infected with HIV and is further tested by a skin test for a hypersensitive response to gp120 envelope antigen.

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(54) Title: IMMUNOTHERAPY IN HIV INFECTED PERSONS USING VACCINES AFTER MULTI-DRUG TREATMENT

(57) Abstract: This invention relates to an improved method of maintaining an immuno-protective response in persons infected with a retrovirus after highly active anti-retroviral therapy.

10/048072

VIRAL LOAD AND CD4+ T-CELL RESPONSES IN UNTREATED/TREATED NYVAC-SIV VACCINATED MACAQUES

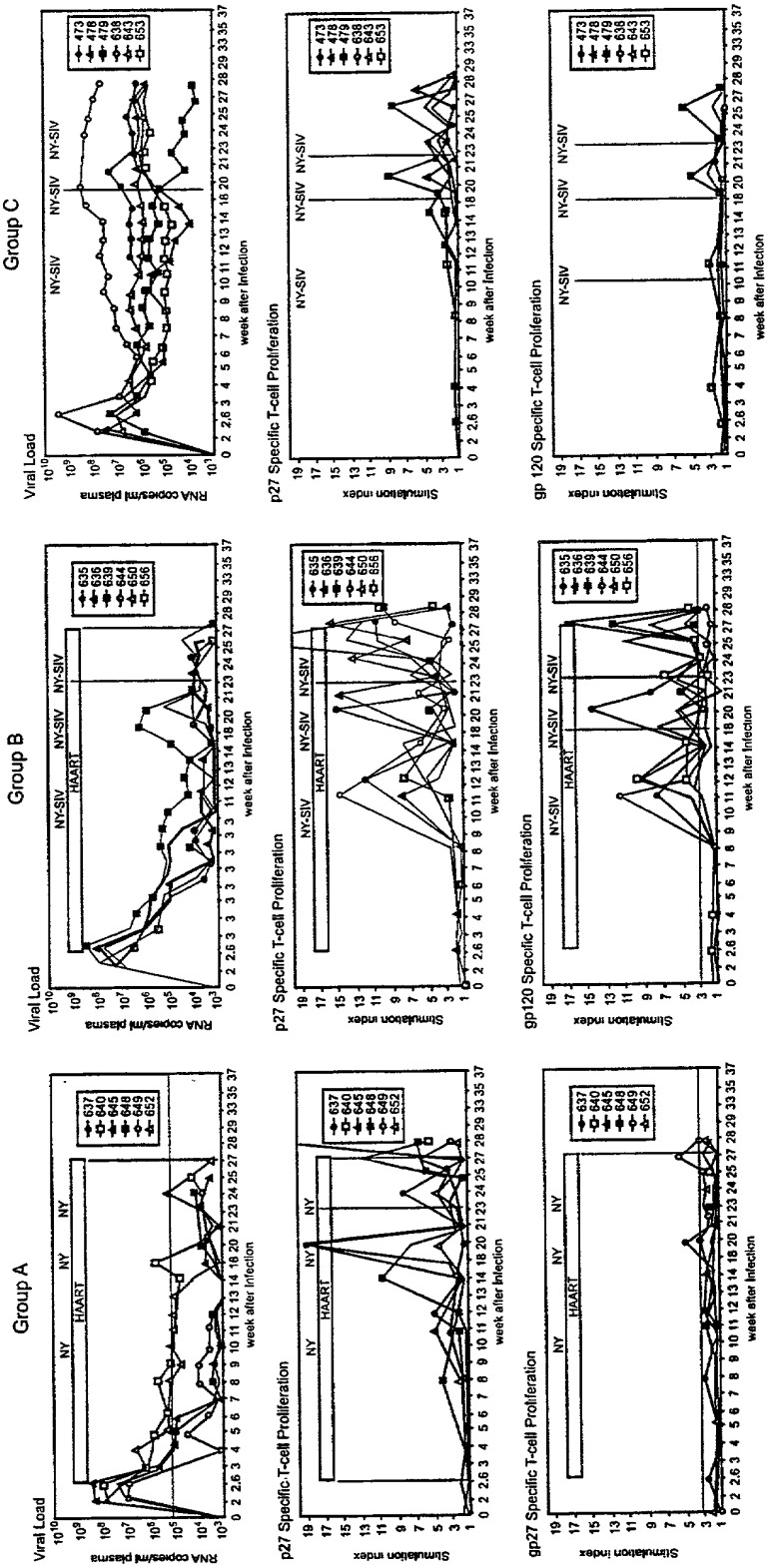
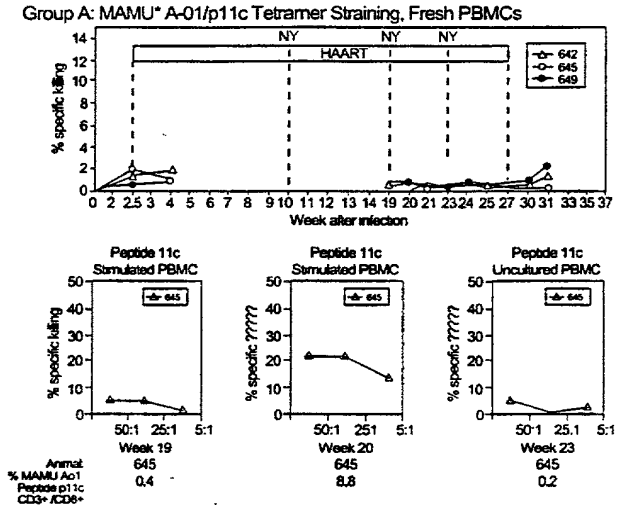
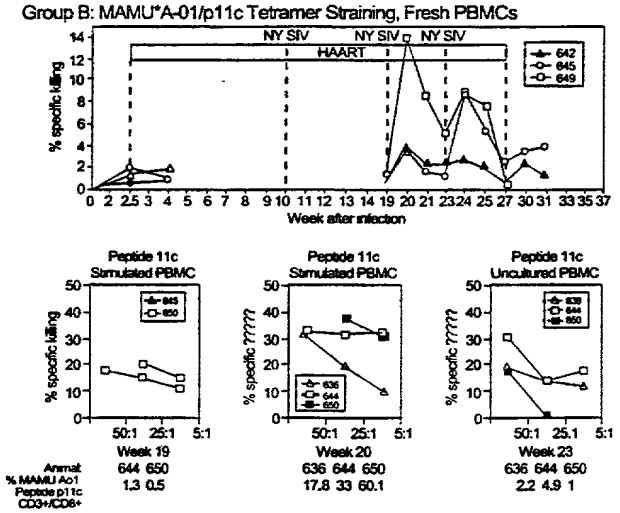


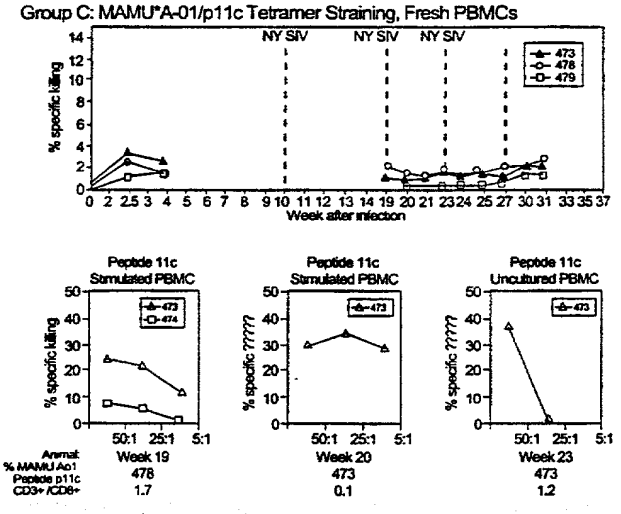
FIG. 1

10/048072

MAMU*A-01/PEPTIDE 11c TETRAMER STAINING, $CD8^+/CD3^+$ T-CELLS- AND PEPTIDE 11c SPECIFIC CYTOTOXIC ACTIVITY IN UNTREATED/TREATED NYVAC-SIV VACCINATED MACAQUES







10/048072

INDUCTION OF PROLIFERATIVE RESPONSE TO p27 gag AND GP120
BYTHE ALVAC-SIV VACCINE IN SIV_{251}-INFECTED, HAART-TREATED MACAQUES

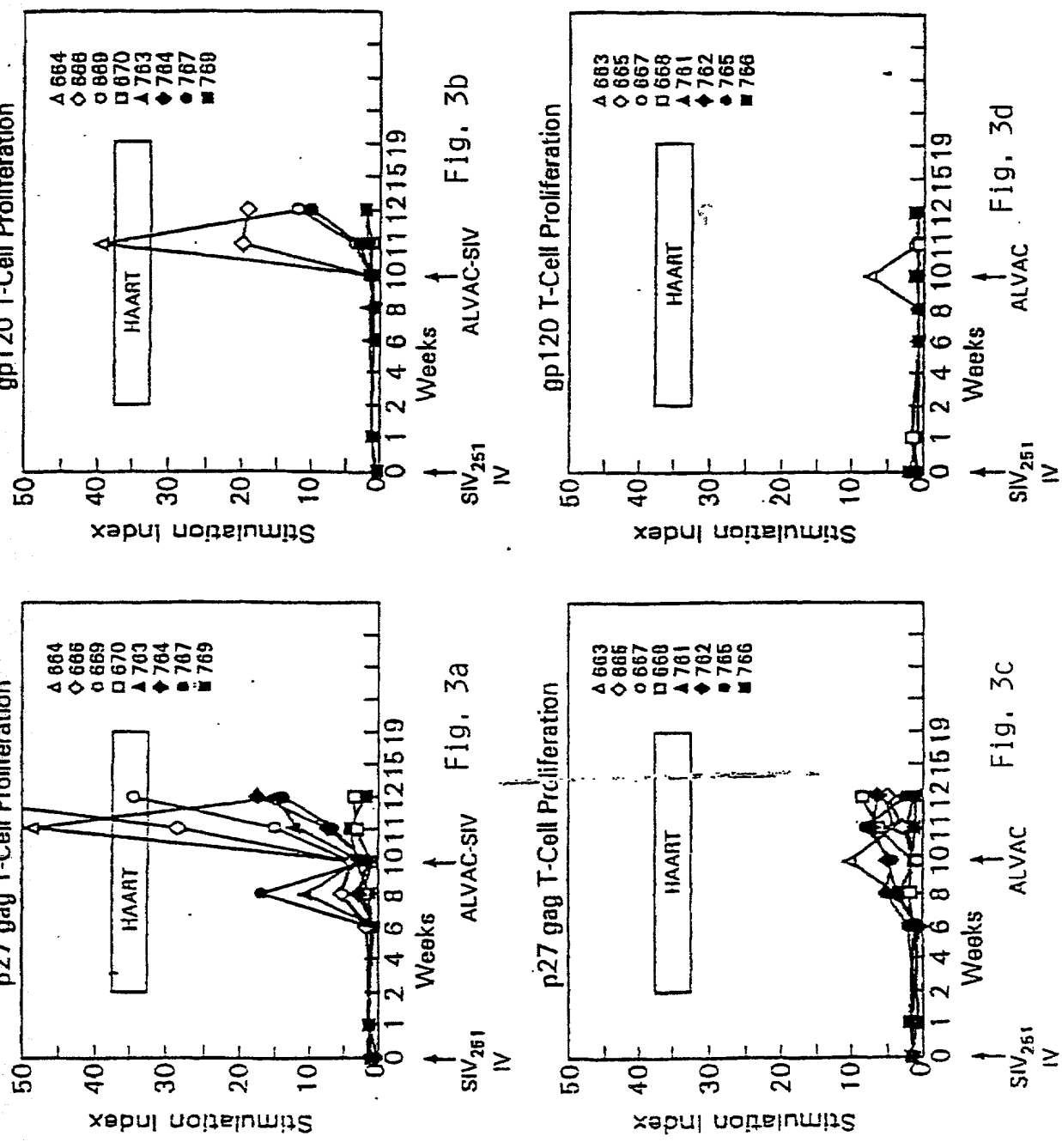


Fig. 3a Fig. 3b Fig. 3c Fig. 3d

Fig. 3

10/048072

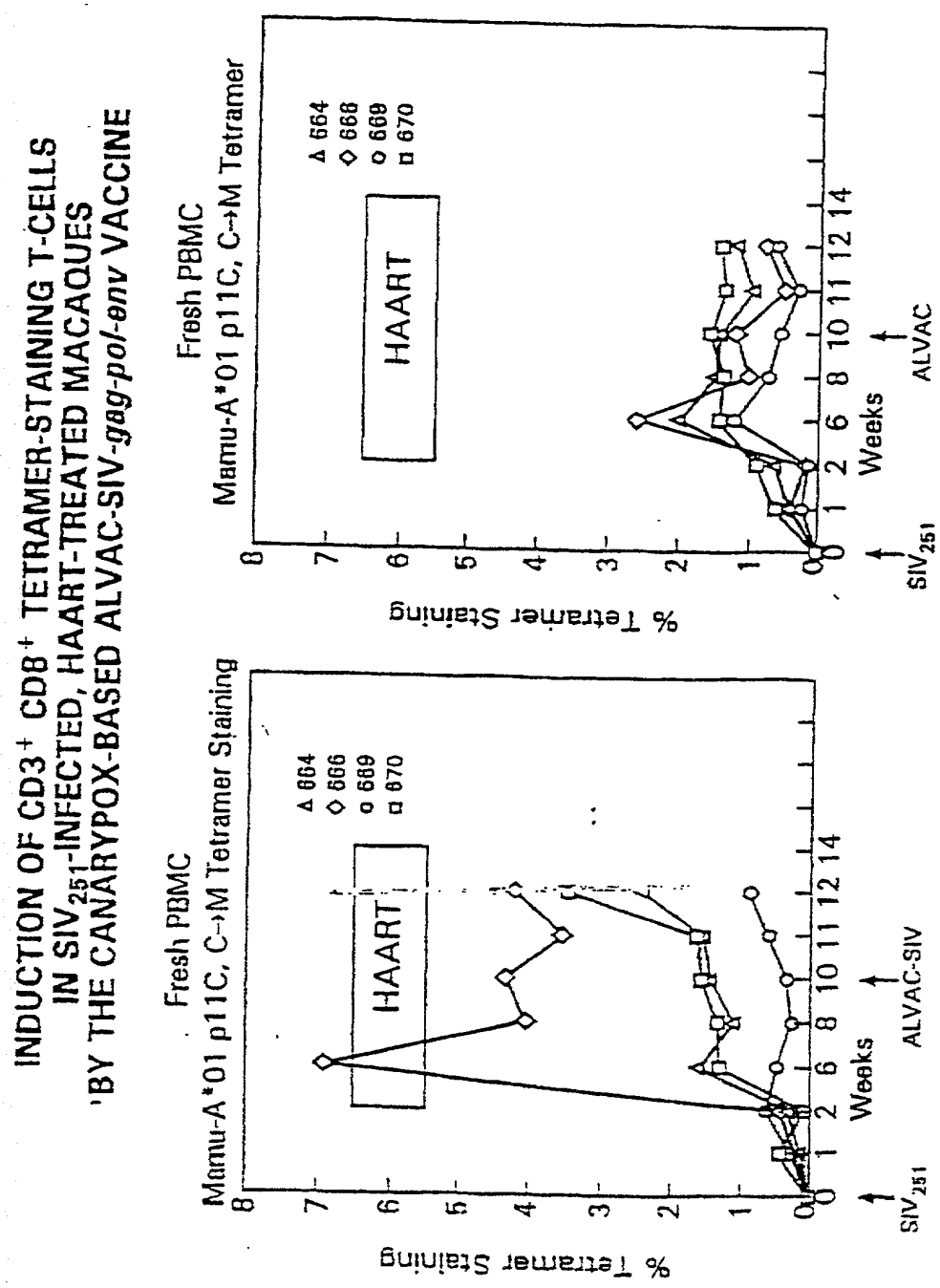


Fig. 4a

Fig. 4b

Fig. 4

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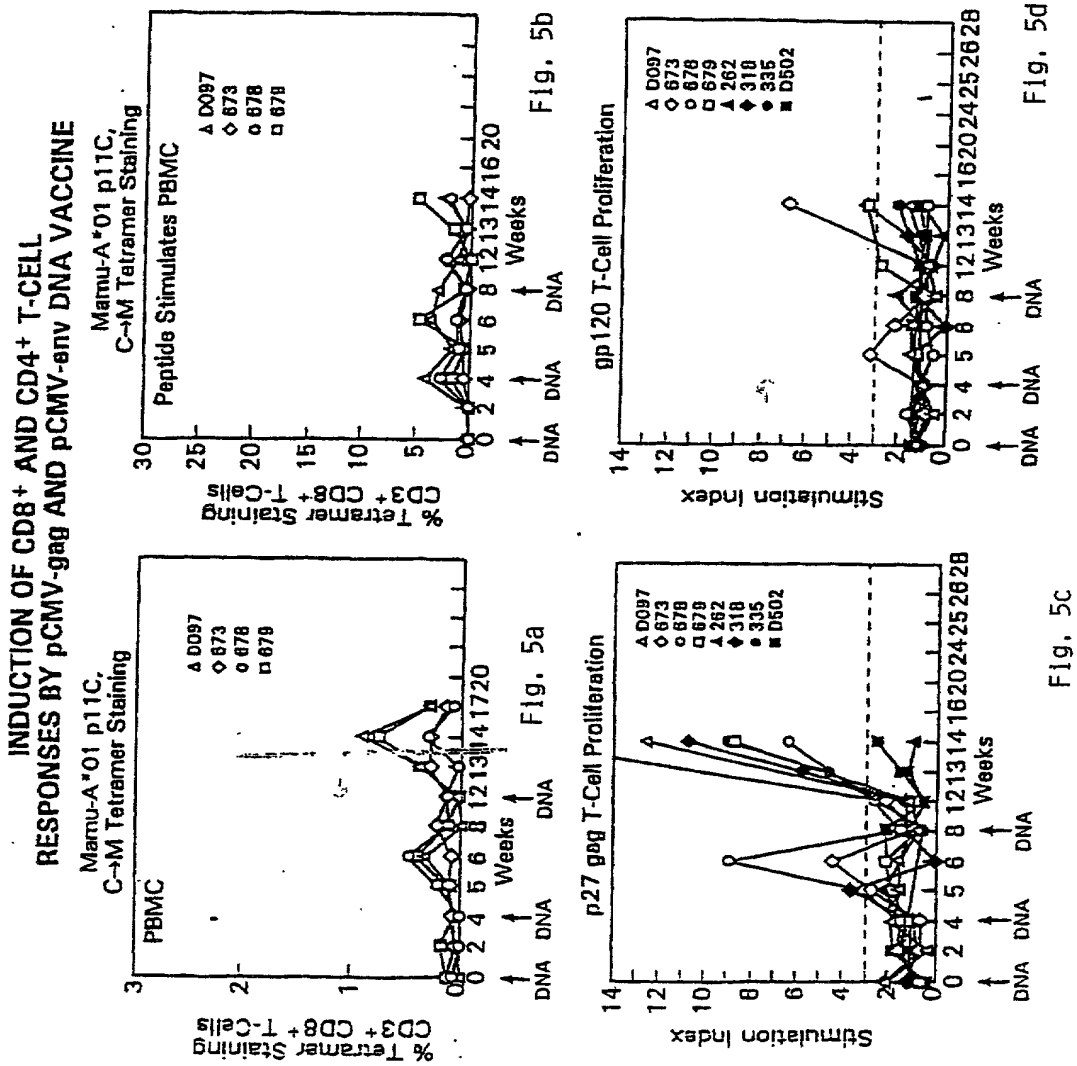


Fig. 5

10/048072

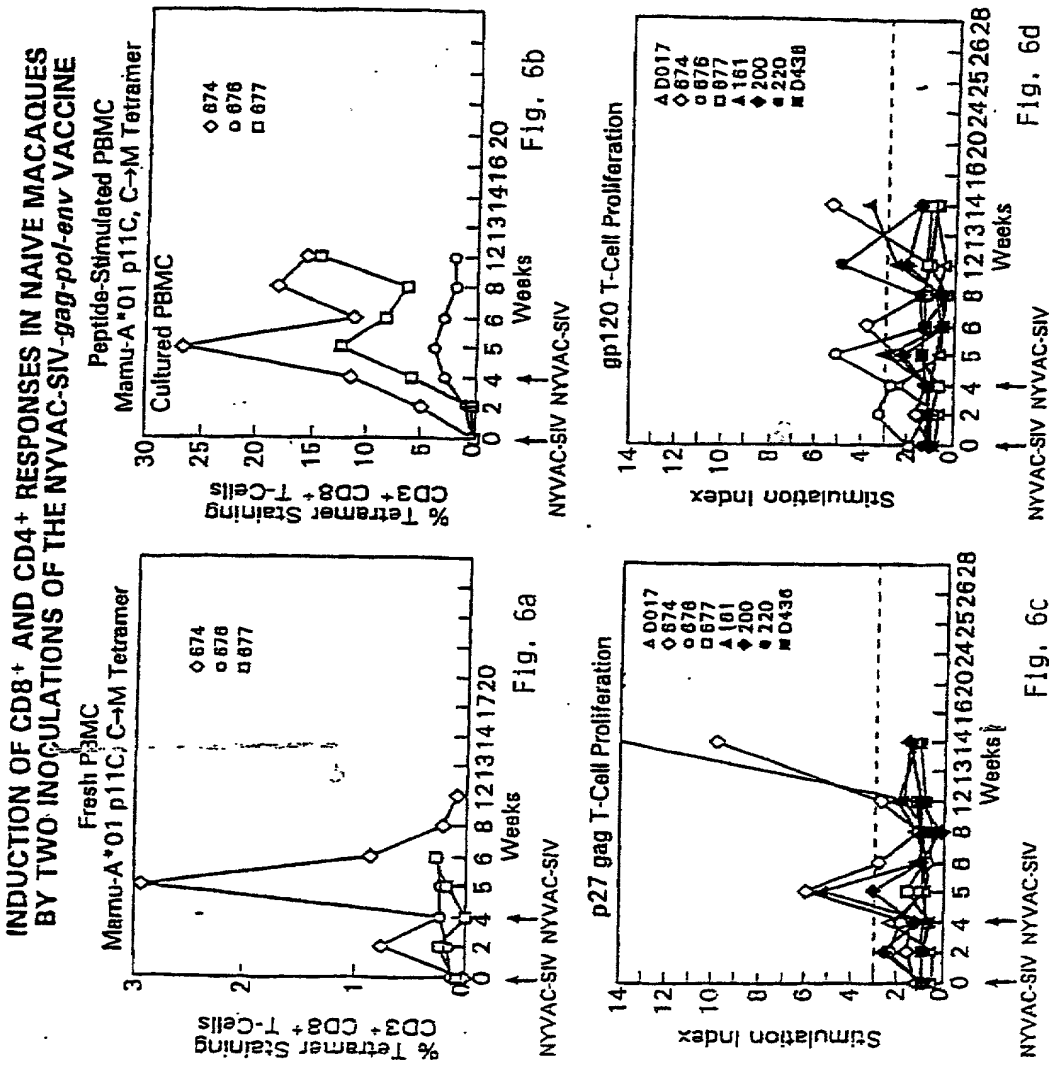
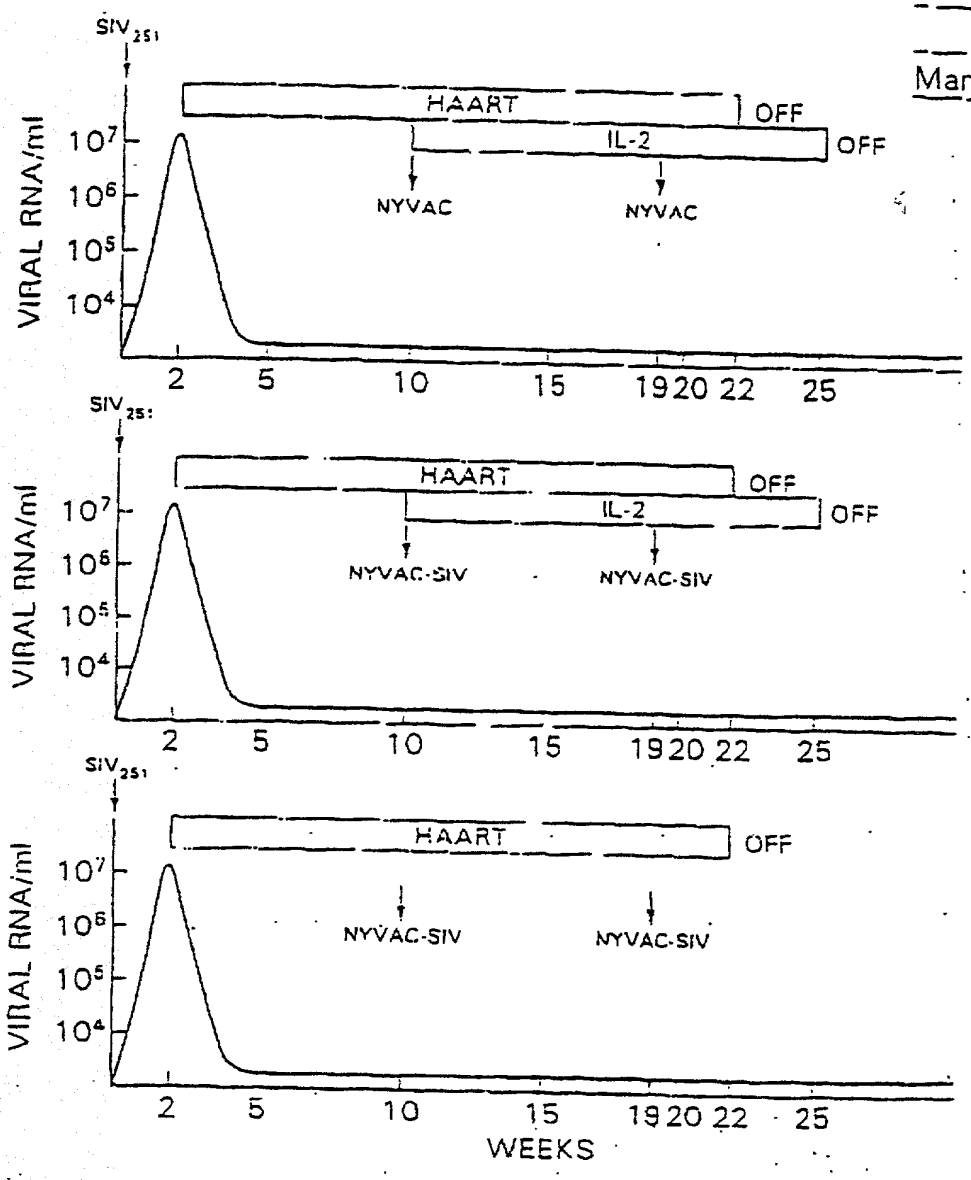


Fig. 6

10/048072

EXPERIMENTAL DESIGN



Animal Number	
Mamu-A*01	Other
6	0
4	2
2	1

Fig. 7

10/048072

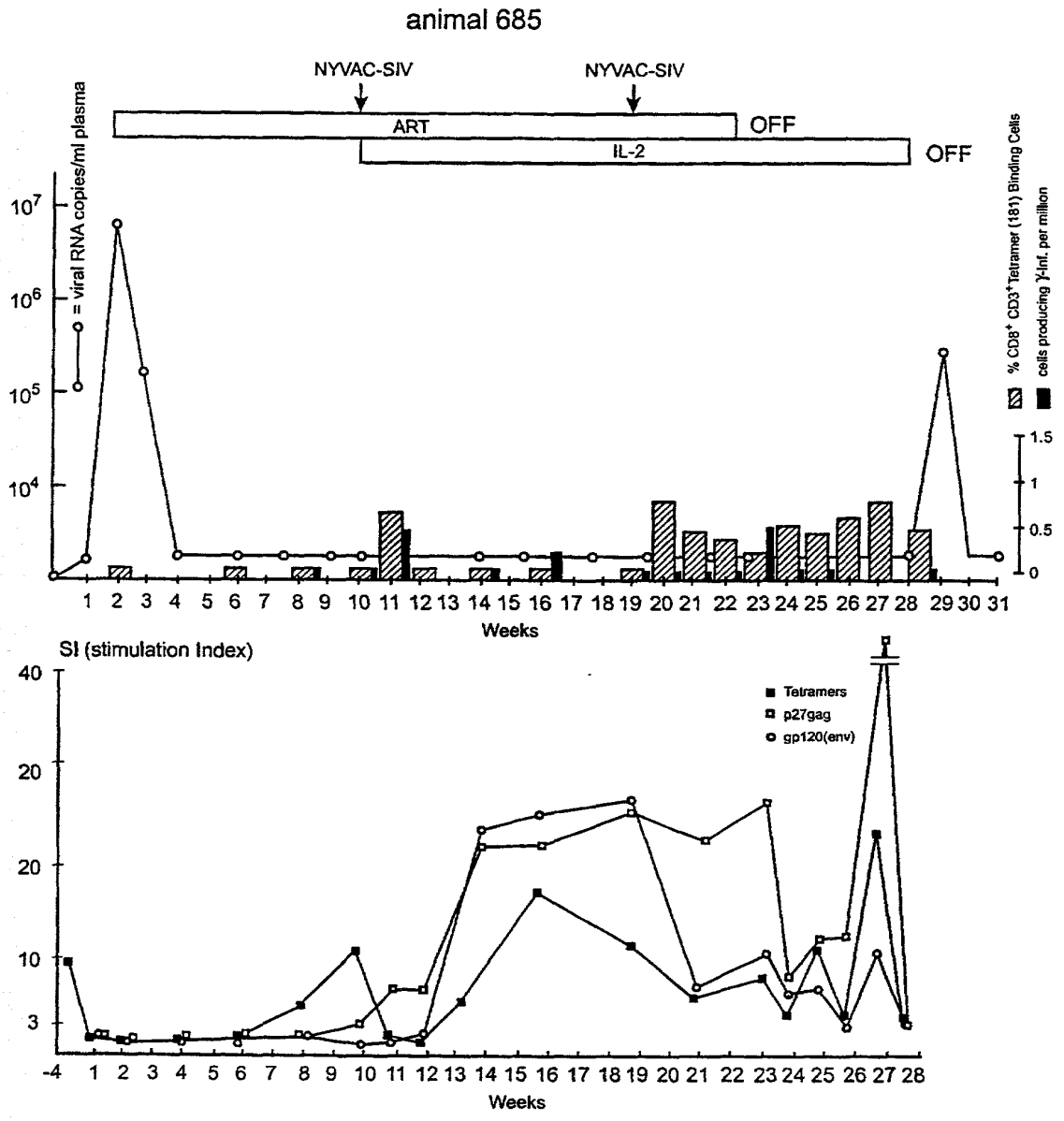


FIG. 8

Attorney Docket No. 015280-400300US
DHHS Ref No. E-212-99/2

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: IMMUNOTHERAPY IN HIV INFECTED PERSONS USING VACCINES AFTER MULTI-DRUG TREATMENT, the specification of which was filed on 27 July 2000 as PCT International Application No. PCT/US00/20641, and which corresponding U.S. National Phase application is filed herewith.

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56. I claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign applications(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Country	Application No.	Date of Filing	Priority Claimed Under 35 USC 119
			Yes ☐ No ☐
			Yes ☐ No ☐

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date
60/146,240	28 July 1999
60/178,989	28 January 2000
60/200,445	28 April 2000

I claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Date of Filing	Status
		☐ Patented ☐ Pending ☐ Abandoned
		☐ Patented ☐ Pending ☐ Abandoned
		☐ Patented ☐ Pending ☐ Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) of the Government of the United States of America as represented by the Secretary of the Department of Health and Human Services, Office of Technology Transfer, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the United States Code, Section 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1	Signature of Inventor 2	Signature of Inventor 3
Genoveffa Franchini	**Zdenek Hel**	**Gene Shearer**
Date 17, 01, 2002	Date Jan 17th. 2002	Date 01-17-02
Signature of Inventor 4	Signature of Inventor 5	
James Tartaglia	**Janos Nacsa**	
Date	Date 1/17/02	

SF 1304569 v1

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the United States Code, Section 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1	Signature of Inventor 2	Signature of Inventor 3
Genoveffa Franchini Date	**Zdenek Hel** Date	**Gene Shearer** Date
Signature of Inventor 4	Signature of Inventor 5	
James Tartaglia Date January 23, 2002	**Janos Nacsa** Date	

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